In Vitro versus *In Vivo* Metabolic Activation of Mutagens

by H. V. Malling* and C. N. Frantz*

Introduction

Mutagenicity testing is beginning to find a place as a factor in evaluation of the safety of compounds to which the human population is exposed. The mutation systems selected for such tests should ideally involve *in vivo* studies with mammals and encompass scoring of a broad spectrum of mutational events including both transmissible chromosome aberrations and point mutations. The frequency of chromosome aberrations obtained after a certain treatment can be assessed quantitatively without prohibitive costs and effort. At the present time there is only one proven system which can be used to screen for specific locus mutations in mammals *in vivo*; the seven specific locus system of Russell. On the other hand there are many microbial systems in which point mutation frequencies can be easily determined quantitatively. It is a known fact, however, that mammals can convert nonmutagenic compounds (promutagens) to highly mutagenic metabolites and that these promutagens are not mutagenic in microorganisms (*1*). In order to combine the drug metabolism of the mammal with the excellence of the microbial systems for measuring point mutations, the host-mediated assay (*1*) and the microsomal system (*2*) were developed. In the host-mediated assay the indicator organism is injected into the animal by one route and the promutagen or mutagen is administered preferably by a different route. The indicator organism is then later recovered and the induced mutation frequency is scored among the recovered cells. In the microsomal system the indicator organism is mixed directly into the reaction mixture containing microsomes, cofactors, and the drug. After a certain reaction time the cells are recovered again and screened for presence of mutants.

Dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) are two chemicals which are nonmutagenic by themselves but which are converted to mutagenic substances by hydroxylation (*3*). DMN is converted to a highly mutagenic compound in the host-mediated assay (*1*) or in the microsomal system (*2*). Both DMN and DEN are potent tissue-specific carcinogens in many animal species. The tumorigenic activity of these compounds seems somewhat related to the presence of the activation enzymes (*4–6*). We felt, therefore, that it was important to correlate the activity of demethylase with the formation of mutagenic metabolites from DMN. The various laboratory test animals respond with great differences to the carcinogenic activity of chemicals. So the choice of test animal for evaluating the carcinogenic activity of a compound is very important. For similar reasons, we felt that it was important initially to evaluate the ability of various laboratory animal strains

*Mutagenesis Branch, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709.

to activate DMN and DEN to mutagenic metabolites both in the host-mediated assay and the microsomal system.

By comparison of the results obtained in the various activation systems and with different indicator organisms, we have attempted to evaluate the relative degree of sensitivity of these systems. As genetic point mutation indicators, we have used the induction of *ad-3* forward mutations in *Neurospora crassa* and the induction of histidine reversions in the histidine-requiring strain G46, of *Salmonella typhimurium.*

The Microsomal System

Preparation of the Microsomes

Sterile technique, sterile glassware, and sterile solutions were used during the preparation of the liver microsomes. The animals were killed by decapitation and bled thoroughly before removal of the liver. After the livers were secured, they were rinsed three times in buffer. Livers from three animals were pooled for each assay. The livers were then homogenized in buffer containing sucrose with a Teflon pistol in a glass tube. The crude microsome preparation was obtained as the supernatant after centrifugation of the liver homogenate at 20,000*g* for 20 min. This is what we have defined in the text as the 20,000*g* supernatant. In order to obtain the purified microsomes, we followed the technique described by Kupfer and Levin (*7*). The following gives a short description of that technique. The livers were prepared and homogenized in the same manner as described above. The homogenate was centrifuged at 10,000*g* for 20 min in order to remove cellular fragments. The supernatant was decanted into a solution containing Mg^{++} and Ca^{++}. In this solution the microsomes aggregated and they were pelleted by centrifugation at 1500*g* for 10 min. The pellet was washed once by suspending it in sucrose and recentrifuging, and it was then resuspended in 1.15% KCl for use. This is what we have defined as the purified microsomes in the text. Microsomal protein was measured accoding to the technique of Lowry (*8*). Microsomes prepared this way correlate well in enzyme content and enzyme reaction with the microsomes pelleted by centrifugation at 100,000*g* (*7, 9*).

Individual Components of the Reaction Mixture

The microsomal reaction mixture is complex. In order to investigate the importance of each component, the formation of mutagenic metabolites was measured in mixtures from which the components were deleted one at a time. Table 1 indicates clearly that they are all necessary. In the case in which N_2 was used instead of O_2, there was no mutagenesis indicating that the formation of the active metabolites was an oxidation process. It was also clear that the formation of the mutagenic metabolites required NADPH which indicated that the activation of DMN was enzymatic. Only in the case where $MgCl_2$ was omitted was there any residual activity, probably due to the fact that the micromomes already contained a certain amount of Mg^{++}.

Microsomal Inhibitors

SKF 525–A is a well-known general microsome inhibitor *(10)*. Its ability to stop the formation of mutagenic metabolites was tested on the 20,000*g* supernatant from homogenized livers of male mice (B6D2F1).

Table 1. Effects of treatment conditions on the frequency of histidine reversion induced in *S. typhimurium* strain TA1530.[a]

Treatment	Survival, %	Number of revertants	Reversions per 10^8 survivors
Complete system	114	916	8.16
Complete system plus N_2 minus O_2	95	2	0.02
Minus NADPH	121	1	0.01
Minus microsomal fraction	126	0	0
Minus $MgCl_2$	109	20	0.19
Minus DMN (control)	100	0	0

[a] The preparation of the microsomal fraction was as described by Malling (*2*).

Without SKF 525–A and with DMN as the promutagen in the normal microsome reaction mixture, 38 revertants per 10^6 survivors were induced in G–46. Upon supplementing the reaction mixture with SKF 525–A to a final concentration of 1*mM*, no increase in the reverse mutation frequency over the control was found, indicating a complete inhibition of the DMN activating enzymes in the microsomes. The same system supplemented with SKF 525–A to give a final concentration of 0.1*mM* had only 3% of the normal mutagenic activity. The experiment confirmed that the formation of the mutagenic metabolites is due to microsomal enzymatic conversion of DMN.

Products of the Metabolism of Aliphatic Nitrosamines

The first step in the metabolic activation of aliphatic nitrosamines is probably hydroxylation of the carbon next to the nitrogen. The subsequent steps may be spontaneous resulting in formation of a carbonium radical and an aldehyde (*11*). In the case of DMN the aldehyde is formaldehyde. This is the product which was determined during enzyme activity studies.

Mutagenicity of Formaldehyde

Although formaldehyde is generally considered to be the nonactive metabolite of DMN, it has nevertheless been shown to be a weak mutagen in several organisms (*12*, *13*). It is, therefore, important to estimate the maximal contribution of formaldehyde to the induced mutation frequency. The mutagenicity of formaldehyde was measured in the microsomal system by use of purified microsomes and formaldehyde as the mutagen. The amount of formaldehyde which is converted depends on the reaction conditions; after 15 min reaction time and with a concentration of 0.1*M* DMN, approximately 1% of the DMN was converted. In order to maximize the conditions for induction of mutations with formaldehyde, it was used in a concentration equal to the maximum experimental conversion of DMN (0.2 μmole ml formaldehyde). *Salmonella typhimurium* G46 was used as the indicator organism in which histidine revertants were scored as mutations. Under these conditions formaldehyde did not give a significant increase over control in reversion frequency.

Kinetics

Table 2 shows the reverse mutation frequency of G46 obtained upon treatment with DMN using various concentrations of the 20,000*g* supernatant in the reaction mixture. It is clear from Table 2 that there is no direct proportionality between the amount of supernatant and the reverse mutation frequency.

By working with a pure microsome preparation, we can simultaneously measure the reverse mutation frequency in one assay and the activity of microsomal demethylase by the formation of formaldehyde. Figure 1 shows the frequency of reverse mutations in G46 after DMN treatment using various amounts of purified microsome in the reaction mixture. The data presented on this Figure 1 are in good agreement with the data presented in Table 2 obtained with the 20,000*g* supernatant. Figure 2 shows the correlation between reverse mutation frequencies and the formaldehyde production.

Table 2. Mutagenicity of DMN in relation to amount of enzyme.[a]

Treatment	Amount of liver (wet), mg	Reversions per 10^6 survivors
Control	100	0.06
	100	119.0
	50	40.0
	25	2.0
DMN (100*mM*)	12.5	0.13
	6.25	0.12
	3	0.05

[a] Livers from three male adult B6D2F1 mice were homogenized in pH 7.5 buffer containing 0.0125*M* MgCl and 0.25*M* sucrose, and centrifuged at 20,000*g*. The supernatant was serially diluted and added to a reaction mixture containing *Salmonella typhimurium*, 4 mg TPNH, and 100 *mM* DMN and incubated 20 min at 37°C under O_2. 100% of the treated bacteria survived.

It is clear from this figure that only after a certain amount of metabolism was there a linear increase in the reverse mutation frequency. It appeared that the active metabolite has to reach a critical concentration or critical total amount before a large number of mutations are induced. After this point was reached, the reverse mutation frequency increased proportionally with the increasing production of formaldehyde. This apparent "threshold" could be due to (a) the presence of a certain number of reactive groups in the reaction mixture which had to be saturated by the active metabolites before any appreciable amount was left over for induction of mutations, (b) a concentration effect on transport across the bacterial cell wall, or (c) certain unique characteristics of the reverse mutation sites in strain G46.

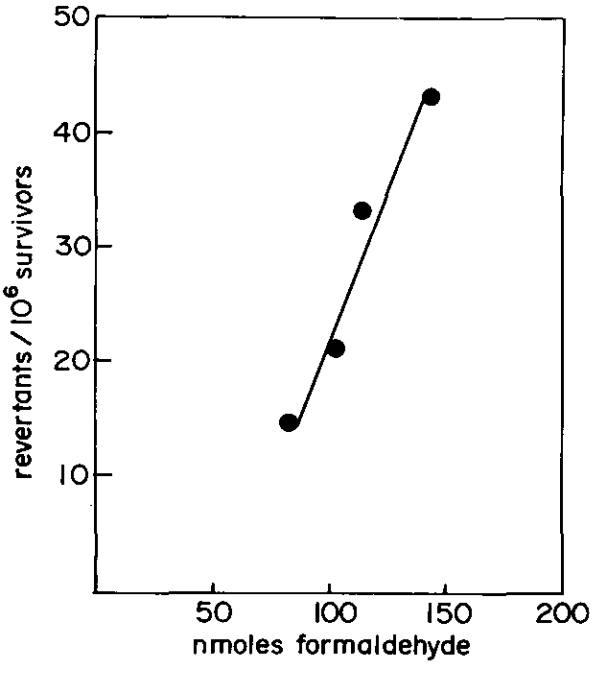

FIGURE 1. Effect of concentration of microsomal protein on the metabolism of DMN to a mutagen. Microsomes were prepared by the calcium precipitation technique from DBA 2/J male mouse livers and added in appropriate amounts to a 15-min incubation containing 1.6 mg TPNH/ml, 100*mM* DMN, pH 7.5 buffer, and *Salmonella typhimurium* His G46.

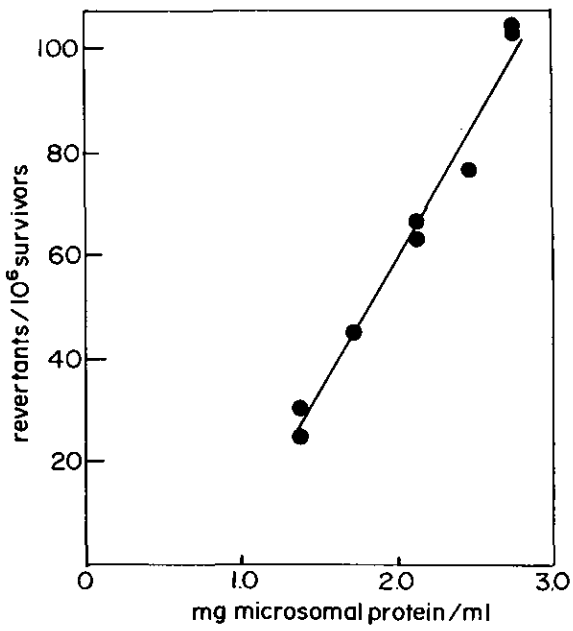

FIGURE 2. Correlation of DMN demethylase activity and metabolism of DMN to a mutagen at varying microsomal protein concentrations. Livers from three male DBA 2/J mice were pooled, and microsomes were prepared by the calcium precipitation technique. Varying quantities of protein were incubated in identical manner for bacterial mutagenesis and DMN demethylase activity (formaldehyde production). The 15-min incubations contained 100*mM* DMN, 1.6 mg/ml TPNH, pH 7.5 buffer, and either water or *Salmonella* in 0.9% saline.

Enzyme Induction

The drug-metabolizing enzymes of the liver can be increased by administering certain compounds to the animal. Phenobarbital and 3-methylcholanthrene (3-MC) are some of the generally used inducers of drug-metabolizing enzymes. Table 3 gives the reverse mutation frequency in strain G46 after treatment with DMN in the microsomal system with the 20,000*g* supernatant. Some of the animals had been pretreated with phenobarbital or 3-MC by intraperitoneal (IP) injections or by prefeeding with butylated hydroxytoluene (BHT). It is clear from these data that pretreatment with all three inducers resulted in an increase in the mutagenic activity of the microsomal system with both DMN and DEN.

Table 3. Effect of enzyme induction of the ability of mouse liver to metabolize DEN and DMN to mutagens.[a]

Inducer	Strain of mice	Mutagen		Revisions per 10^8 survivors	
		Type	Concn, *mM*	Noninduced	Induced
Phenobarbital	B6D2F1	DMN	180	177	378
Control				0.03	0.04
3-MC	B6D2F1	DMN	180	129	152
Control				0.02	0.03
BHT	101;C3F1	DMN	45	27	62
Control				0	0.01
Phenobarbital	B6D2F1	DEN	200	0.53	0.93
Control				0.03	0.04
3-MC	B6D2F1	DEN	200	0.34	0.57
Control				0.02	0.03
BHT	101;C3F1	DEN	45	0.13	0.32
Control				0.05	0.05

[a] Livers from at least three male mice were pooled to obtain each mutant frequency. The 20,000*g* supernatant of the liver homogenate (2 ml solution/liver) was incubated with 4 mg TPNH, *Salmonella typhimurium*, and DMN or DEN for 20 min. Sodium phenobarbital was given IP, b.i.d., doses of 80/mg/kg-day for 3 days, the last injection 12 hr prior to sacrifice. Age-matched controls were given corresponding injections of saline. 3-Methylcholanthrene, 0.5 mg in 0.5 ml olive oil, was injected IP 24 hr prior to sacrifice; age-matched controls received 0.5 ml olive oil. BHT was fed to animals as 0.75% of diet for 14 days.

ad–3 Mutation System in *Neurospora crassa*,

This mutation system is based on detection of forward-mutations in two adenine genes, *ad-3A* and *ad-3B* (*14*). Mutants in the two genes block the purine biosynthesis in two sequential steps, and lead to accumulation of purple pigments in the mycelium. The mutants are detected as purple colonies among white nonmutated colonies. The colonies are grown submersed, and it is easy to screen 50×10^6 colonies in a day. Neurospora is normally haploid but forms heterokaryons. A heterokaryon heterozygotic for the two adenine-genes, *ad-3A* and *ad-3B*, is white. The heterokaryons mimic the diploid cells in many ways. Purple *ad-3* mutations induced in such a heterokaryon can be point mutations in either of the two genes or deletion of either one or both of the genes. The *ad-3B* mutants can either be noncomplementing or have a polarized or nonpolarized complementation pattern. The *ad-3A* mutants do not show complementation. Work with the reverse mutation frequencies in various mutants induced by a series of specific chemical mutagens and with the complementation response of the mutant shown that there are many different mutational sites in both loci (*15–17*).

From growth studies it is known that the mutants recovered in de Serres' forward mutations system ranged from mutants totally dependent on adenine to mutants which can grow with wild-type rate on minimal medium (*18*). We can, therefore, conclude that in this system a broad spectrum of mutational damage can be detected.

It has previously been established that DMN and DEN do not induce mutations in directly treated *Neurospora* conidia. However, in a chemical system which mimics the liver metabolism of these two compounds (*3*), both DMN and DEN are mutagenic. Thus, the active metabolites of DMN and DEN are mutagenic in *Neurospora*.

The *Salmonella typhimurium* His G46 Locus

Salmonella normally do not require histidine to grow. However, Ames (*19*) has iso-

Table 4. Induction of *ad-3* mutations in *Neurospora* and histidine reversion in *S. typhimurium* (G46) in the host-mediated assay after treatment of the mice with DMN.

Organism	DMN, mg/kg	Mutations per 10^6 survivors
Neurospora	100	0
Salmonella His G46	100	1.94

lated a number of mutant strains which require histidine. Strain G46 is such a mutant strain, and it can be reverted to histidine independence by a base-pair substitution. Thus, detection of mutagenicity using this single strain is restricted to a limited number of sites in the DNA, in which changes result in a reverse mutation.

Comparison of Indicator Organisms

Table 4 shows the frequency of *ad-3* mutations obtained among *Neurospora* conidia injected into the peritoneal cavity of mice when the mice were treated with DMN and the frequency of histidine revertants induced in *S. typhimurium* G46 under similar conditions.

It is clear that the reversion sites in G46 are much more sensitive than the *ad-3* loci of *Neurospora*. This is a surprising result, since in G46 the mutations induced in a few codons are being measured, whereas in *Neurospora* many mutational sites on two genes are being screened. Two possible explanations are: (a) the active metabolic product of DMN may not penetrate as well into *Neurospora* as into *Salmonella* or (b) the particular mutational site in G46 may represent a "hot spot" for the particular type of alkylation produced by the active metabolite of DMN.

Comparison of Host-Mediated Assay and Microsomal Assay

Table 5 shows the results obtained in an host-mediated assay with the use of *Neurospora* as the indicator organism and treatment of the animal with either DMN or DEN. Several routes of administration were used; the conidia were injected either IP or into the tail vein (IV). With IV injection a portion of the conidia are trapped in the liver and lungs of the animal. Biopsies have shown the conidia well dispersed.

Upon assay of the promutagen DEN, no effect was found when the *Neurospora* were injected IP, and a slight increase in mutation frequency over control was observed in conidia injected IV and recovered from the liver. Similarly, DMN did not increase the mutation frequency of IP conidia above the control and in *Neurospora* injected IV and isolated from the liver, DMN increased the mutant frequency dramatically.

Table 5. Effect of route of administration of conidia and DMN or DEN on mutation induction in *Neurospora* conidia in the host-mediated assay.

Experiment	Treatment	Pro-mutagen dose, mg/kg	Route of administration		Number of *ad-3* mutations	Mutations per 10^6 survivors
			Conidia	Chemical or saline		
12–187	*In vitro* control	0	—	—	0	0
	DEN, in mice	200	IP	IM	1	0.31
12–195	In mice, liver	0	IV	IP	5	0.65
	DEN, in mice, liver	150	IV	IP	14	1.74
12–187	*In vitro*, control, 37°C	0	—	—	0	0
	Control, mice	0	IP	IM	0	0
	DMN, mice	100	IP	IM	1	0.40
12–201	*In vitro*, control, 37°C	0	—	—	0	0
	Control, mice, liver	0	IV	IM	0	0
	DMN, mice, liver	75	IV	IM	528	286

An explanation of these results must take into account both anatomy and metabolism. We suggest that the highly reactive metabolites of DMN and DEN are extremely short-lived and cannot easily survive transport from the liver, where the metabolites are primarily formed, to the peritoneum. However, when the conidia are sitting in the liver, they may easily come into close contact with the active metabolites.

It is difficult to make comparisons of data obtained from the host-mediated assay with that obtained from microsomal mutagenesis assays. In the host-mediated assay, the survival of the host is the determining factor for maximum dose; in the microsomal system some efficiency of metabolism may be lost. However, the magnitude of the differences does allow for some comparison of the assay techniques in terms of their usefulness in screening for promutagens.

The mouse host-mediated assay with G46 with 3 hr of IP incubation and a dose of 100 mg/kg DMN (equal to a concentration of 1.3 *mM* DMN in the mouse assuming equal tissue distribution) induced 1.94 reversions per 10^6 surviving bacteria. In the mouse microsome system, 30 min of incubation with 180 *mM* DMN induced 200 reversions per 10^6 survivors (100% survival). The data indicate that the peritoneal cavity is a very ineffective place to measure labile mutagenic metabolites formed in the liver. The same conclusion was reached above when the mutagenicity of DMN in *Neurospora* conidia in intraperitoneal and intrahepatic (IH) locations were compared.

Nevertheless, the inefficiency of using the microsomal system to form mutagenic metabolites is clearly demonstrated in the next series of experiments. In the mouse microsome system, under the same conditions under which DMN induced 100–200 revertants per 10^6 survivors in bacteria, only 5 *ad-3* mutations per 10^6 survivors were found in *Neurospora* (Ong, personal communication). In *Neurospora* conidia injected IV and recovered from the liver, the *ad-3* mutation frequency is 250 per 10^6 survivors.

In summary of this discussion of alternative assay methods, we are looking for a mutagenesis assay which is the most sensitive in the detection of mammalian metabolism of a promutagen to mutagenic but labile products. The *Salmonella* G46 data indicate that an *in vitro* microsomal system is more sensitive than the intraperitoneal host-mediated assay, and the *Neurospora* data indicate that the intrahepatic host-mediated assay is even more sensitive than the microsomal assay.

Species and Strain Differences

Rats and Mice

Table 6 gives the results of using DEN and DMN in the liver microsome system when the microsomes were obtained from either mice or rats. In general, there is good agreement between the *in vitro* system and the host-mediated assay; DEN does not show very high mutagenic activity in either system (Table 7). One surprising result is that, in comparison with the results from the host-mediated assay, DMN did not show a very high mutagenicity in the rat liver microsome system. A possible explanation for that is that in the host-mediated system,

Table 6. Induction of histidine reversions in G46 (*S. typhimurium*) in the microsomal system (from liver).

Animal	Treatment	Promutagen, *mM*	Number of revertants	Reversions per 10^8 survivors
Rat	Control	0	11	0.06
Rat	DEN	45	8	0.05
Rat	DMN	45	20	0.11
Mouse	Control	0	9	0.06
Mouse	DEN	45	51	0.31
Mouse	DMN	45	1042	6.2

Table 7. Induction of histidine reversions in G46 under host-mediated assay conditions.

Experiment	Species	Treatment	Promutagen, mg/kg	Number of revertants	Reversions per 10^8 survivors
27–112	Rat	Control	0	7	0.02
	Rat	DMN	200	193	1.01
27–116	Mouse	Control	0	0	0
	Mouse	DMN	25	2153	0.58
	Mouse	DMN	200	2527	1.41
27–118	Rat	Control	0	4	0.0037
	Rat	DEN	400	8	0.0068
27–119	Mouse	Control	0	3	0.0012
	Mouse	DEN	400	24	0.0070

one utilizes the total rat liver, which is much bigger than the mouse liver, whereas in the microsomal system the same amount of liver from both mice and rats was used.

Mouse Strains

In evaluation of the safety of chemicals for the human population an everpresent dilemma is the selection of the proper mammalian species and within the species the strain. As noted previously in the introduction, great differences exist between rats and mice. Table 8 gives the comparison between mouse strains C57BL/6, DBA2/J, and the hybrid of the two strains, B6D2F1. Strain C57BL/6 has lower activity than DBA2/J, which has the same activity as the F1 hybrid between the two strains. Table 9 gives similar results obtained with another series of strains and a hybrid. It is clear from these data that C3H (Oak Ridge) has a considerably higher activity than the hybrid B6C3F1, and this is in strong contrast to the previous table where the hybrid equaled the top strain of the cross in activity. If DBA2/J can serve as basis for comparison, then there is an eightfold difference in activity between C3H and C57BL/6. On the other hand, Table 9 shows clearly that similar differences did not exist for activation of DEN. We think these data indicate the enzymes activating DMN and DEN are different.

S. typhimurium G46 reverts after treatment in the microsomal systems mixed with DEN at a much lower rate than when mixed with DMN. Since the mutation activity is

Table 8. Variation among mouse strains in the metabolism of DMN to a mutagen.[a]

Mouse strain	DMN, *mM*	Reversions per 10^8 survivors
C57B1/6	180	19
DBA 2/J	180	38
B6D2F1 (hybrid)	180	35

[a] Three animals of each strain, which had been housed and fed under similar conditions for at least 2 weeks and who were matched for age, were pooled to determine each mutant frequency. Livers were homogenized in 2.5 ml solution/liver, and equal amounts of 20,000*g* supernatant were incubated with 180 *mM*, DMN, 4 mg TPNH, and *Salmonella typhimurium* for 20 min.

Table 9. Variation among mouse strains in ability to metabolize DMN and DEN to a mutagen.[a]

Mouse strain	Compound	Promutagen, *mM*	Reversions per 10^8 survivors
C3H (Oak Ridge)	DMN	90	41
DBA 2/J	DMN	90	10
B6C3F1	DMN	90	15
DBA 2/J	—	0	0.02
C3H	DEN	200	0.60
DBA 2/J	DEN	200	0.51
B6C3F1	DEN	200	0.56

[a] Pools of three mice from each strain were used. The livers were homogenized in 4 ml/gm liver of buffered solution and centrifuged at 20,000*g*. The supernatant was incubated 20 min with *Salmonella* G46, 4 mg TPNH, and the promutagen.

scored as revertants, it could be an expression of the specificity of the reverse mutation mechanism and not a difference in activity of the enzymes. However, in a host-mediated assay with Neurospora as indicator organism in which the conidia were injected IV and recovered from the liver, the *ad-3* mutation frequency among the conidia after DMN treatment of the animal was 500 times higher than after treatment of the animal with DEN. Thus, the difference in the mutagenicity of DMN and DEN are consistent with different indicator organisms, and we can conclude that the results obtained with G46 probably are due to difference in enzyme activity and not due to specificity in reversion mechanism for active products of these two chemicals.

In line with these observations, the data in Table 10 shows that there is very little increase in the reversion frequency when the DEN concentration is varied from 25 *mM* to 200 *mM*.

Table 10. Mutagenicity of DEN in various concentrations in a microsomal preparation.[a]

DEN concentration, *mM*	Survival, %	Reverse mutations per 10^8 survivors
25	79	0.51
50	95	0.32
100	68	0.60
200	52	0.81
0	100	0.15

[a] Livers from three male B6D2F1 mice were homogenized in 2 ml of buffered solution per gram liver. The 20,000*g* supernatant was incubated with 4 mg TPNH, DEN, and *Salmonella* for 20 min.

Purified Microsomes and Strain Differences

A better understanding of the basis for the strain differences and the difference between induced and noninduced animals can be obtained by using purified microsomes. The two most extreme mouse strains with respect to their ability to form mutagenic metabolites were selected for the experiment. They were C57BL/6 (the low activity strain) and C3H (the high activity strain). 3-MC was used as an enzyme inducer. In Table 11 the amount of purified microsomes per gram liver is shown. It increased dramatically after induction by 3-MC in C57BL/6 mice. The mutagenicity of DMN with purified microsome was measured using the same amount of protein. It is clear from Table 11 that in C57BL/6, mice induced by 3-MC the same amount of purified microsomes contain a higher amount of demethylase activity than in noninduced C57BL/6 mice and that in strain C3H the

Table 11. Effect of 3-methylcholanthrene enzyme induction on livers of C3H and C57B1/6 mice in terms of microsomal protein yield, DMN demethylase activity, and metabolism by the microsomes of DMN to a mutagen.[a]

Mouse stain	Inducer	Liver, g	Purified microsomes, mg	Microsomes, mg/g liver	Demethylase activity nmole HCHO/ mg protein-min	Reversions per 10^8 survivors
C57B1/6	Control	4.6	40	8.7	2.66	13.8
C57B1/6	3-MC	3.9	65	16.6	4.66	42.9
C3H	Control	4.9	69	14.1	3.53	20.3
C3H	3-MC	4.6	75	16.3	3.88	31.8

[a] All mice were males matched for age and raised in the same environment for at least 2 weeks prior to sacrifice. 3-Methylcholanthrene, 0.5 mg in 0.25 ml olive oil, was injected IP 24 hr prior to sacrifice; controls received 0.25 ml olive oil IP. Pools of three animals were used to determine each data point. Microsomes were prepared according to the method of Kupfer and Levin (*7*); protein was measured by the Lowry technique (*8*). Identical 15-min incubations were done for formaldehyde production and bacterial mutagenesis, except no bacteria were present in the demethylase assay. Incubation contained 100*mM* DMN, 1.6 mg microsomal protein/ml, 1.6 mg TPNH/ml, pH 7.5 buffer, and either water or *Salmonella typhimurium* in 0.9% saline.

demethylase activity per milligram protein is almost the same.

Another question raised is the correlation between mutagenic activity and demethylase activity when different strains are considered and they are induced with 3-MC. By plotting in the data obtained from under these various conditions on the curve describing the relationship between demethylase activity and reversion frequency (Fig. 3), a very good agreement was demonstrated between demethylase and reversion frequency independent of the variation with respect to strain and to induction with 3-MC.

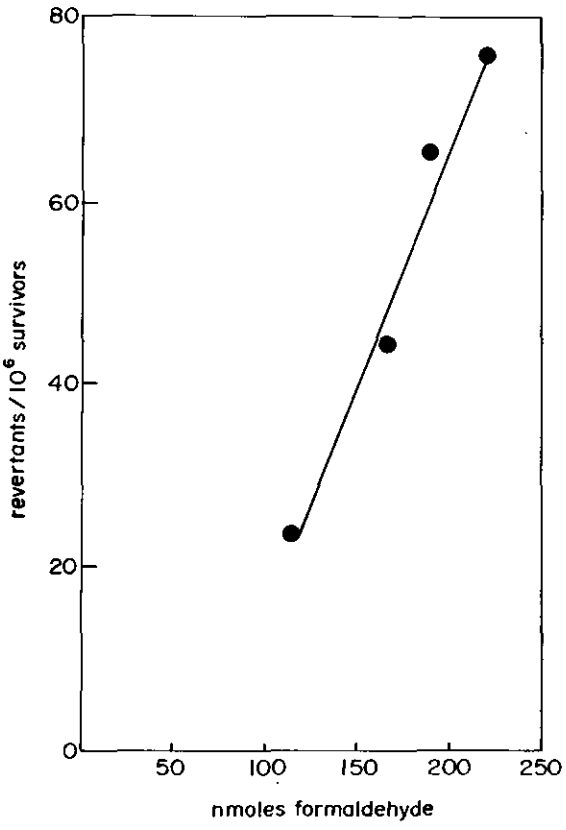

FIGURE 3. Correlation of microsomal DMN demethylase activity and microsome metabolism of DMN to a mutagen. Two related strains of mice with and without 3-methylcholanthrene enzyme induction. This figure is an illustration of data from Table 11.

It is interesting to note that the mouse strain with the lowest basic demethylase activity is the strain which is most inducible, and when both are induced, this relationship between strains in terms of demethylase activity is reversed.

Discussion

Both DMN and DEN require metabolic conversion before becoming mutagenic. Both compounds probably form highly labile active intermediates. The degree of reaction with DNA is likely to depend on the presence of activating enzymes. After treatment of an animal with DMN, the DNA of the various tissues are alkylated to greatly different degrees. The testes are among organs alkylated the least. After treatment of mice with MMS or DMN in equal doses the amount of alkylation measured as 7-methylguanine in the DNA of the testis is, respectively, 0.05% and 0.0007% (*4*). It seems unlikely, therefore, that DMN will give any detectable increase in the mutation frequency of the male germinal cells. Why then study the mutagenesis of DMN at all?

The design of most experiments to study chemical mutagenesis in mammals involves acute or subacute treatments of the mammal. We have no data which would tell us whether there is an accumulation of point mutations during chronic administration of chemical mutagens to mammals. This is, however, the most prevalent situation for exposure of man to chemicals. One of the important questions in extrapolating the test data to man is: What relationship is there between the genetic effects of approximately 30 years exposure of man to low concentrations of a certain chemical and the mutation data obtained after acute treatment of a mammal with the same chemical?

Ideally, we may want to base the safety evaluation of a compound on mutation systems based on detection of germinal mutations *in vivo* in mammals. The question would then be: Does that compound give a significant increase in the frequency of mu-

tations? As mentioned earlier, it is unlikely that DMN would give any detectable increase in the number of germinal mutations when the testing is carried out in the standard way. Maybe therefore, in the safety evaluation of a chemical, it would be preferable to take a more conservative posture and base the evaluation on seeking an answer to the following question: Can the mammal convert the chemical under test to a mutagenic metabolite? If so, there is reason for caution. It is in the attempt to answer the question that the host-mediated assay and the microsomal system are potentially most useful.

In assessment of the mutagenicity of environmental compounds it is important that the test system is as relevant to man as possible. The liver microsome system, with the use of human liver microsomes and human cells, represents such a possibility. Most pharmacologists, however, would agree that the liver microsomal system can give false negative results due to lack of, for instance, the right coenzyme or other conditions which are required for the biochemical reactions to proceed.

Summary

In summary, we have explored a number of factors related to mammalian activation of promutagens. We define DMN and DEN as promutagens needing mammalian metabolism to form highly mutagenic products. For DMN the activation is microsomal, needs some cofactors, and the mutagenic activity of the product is directly correlated with the metabolic formation of formaldehyde with and without induction and across strains of mice. Formaldehyde does not contribute to the mutagenic activity of the reaction products.

Selection of a mammalian metabolizer of promutagens should be made with the variability between species and strains in mind. Induction of microsomes may increase sensitivity in a given strain.

At least with DMN and DEN, *Salmonella* G46 is far more sensitive than *Neurospora* as an indicator organism.

Finally, the proximity of the indicator organism to the major site of metabolism is related to mutagenic activity in terms of orders of magnitude. The microsomal assay is a few orders of magnitude more sensitive than the IP host-mediated assay, and the intrahepatic host-mediated assay is a few orders of magnitude more sensitive than the *in vitro* microsomal system.

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